

RECOMBINANT ANTI-CD40 ANTIBODY AND USES THEREOF

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RECOMBINANT ANTI-CD40 ANTIBODY AND USES THEREOF

1. FIELD OF THE INVENTION

5 The present invention relates to methods and compositions for the treatment of diseases and disorders, including cancer, inflammatory diseases or disorders and diseases or disorders of the immune system, comprising administering a CD40 binding protein which enhances binding
10 of CD40 ligand to CD40. CD40 binding proteins include recombinant/variant forms of monoclonal antibody S2C6 and derivatives thereof.

2. BACKGROUND OF THE INVENTION

15 CD40 is a cell surface phosphorylated glycoprotein that is expressed on a variety of cell types, including B cells, B cell malignancies, follicular dendritic cells, basal epithelial cells, and carcinomas. CD40 binds CD40 ligand ("CD40L"). CD40L is expressed on activated T cells during inflammation and cancer (Younes et al., 1998, Br. J. Haematol. 100:135-141; for a review see Grewal and Flavell,
20 1998, Annu. Rev. Immunol. 16:111-135). The interaction of CD40 with CD40L results in B cell activation and proliferation of normal B cells; however CD40-mediated signaling in B cell-derived tumor lines can result in
25 activation-induced cell death. The strength of the activation signal is key to activation-induced tumor cell death (Grafton et al., 1997, Cell. Immunol. 182:45-56). Therefore, compositions and methods for increasing receptor-ligand interaction and strength of activation signal between
30 CD40 and CD40L would be of great value in treating disease.

2.1 CD40 AND CD40 LIGAND

CD40 is a member of the TNF receptor superfamily. This family includes TNFrII, CD40, CD30, LMP-1, LTBr, ATAR, OX-40
35 and 4-1BB receptors. CD40 is constitutively expressed on B-lymphocytes, macrophages and dendritic cells and is induced

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by cytokine activation on fibroblasts, endothelial cells and epithelial cells (Van Kooten and Banchereau, 1997, Curr. Opin. Immunol., 9: 330-337). CD40 has also been shown to be highly expressed on many human carcinomas including lung, bladder, gastric, breast and ovarian cancers (Stamenkovic et al., 1989, EMBO J. 8:1403-1410).

The ligand for CD40 is a membrane protein that is expressed on activated T cells. Receptor binding of CD40L results in CD40 multimerization, the generation of activation signals (for antigen presenting cells such as dendritic cells, monocytes and B cells) and the generation of growth and differentiation signals (for cytokine-activated fibroblasts and epithelial cells). CD40 signals are transduced from the multimerized receptor via recruitment of a series of TNF receptor-associated factors ("TRAFs") (Kehry, 1996, J. Immunol. 156:2345-2348). Subsets of TRAFs interact differentially with TNF family members, including CD40, to provide stimuli to a wide variety of downstream pathways. TRAF1 and TRAF2 are implicated in the modulation of apoptosis (Speiser et al., 1997, J. Exp. Med. 185:1777-1783; Yeh et al., 1997, Immunity 7:715-725). TRAFs 2, 5, and 6 participate in proliferation and activation events, including NF-kB and c-Jun N-terminal kinase activation. In normal B cells, binding of CD40 recruits TRAF2 and TRAF3 to the receptor complex and induces down-regulation of other TRAFs (Kuhune et al., 1997, J. Exp. Med. 186: 337-342). The effects of CD40 binding are also dependent on membrane density (De Paoli et al., 1997, Cytometry 30:33-38). Importantly, unlike the proliferative response seen with normal primary B cells, CD40 binding on neoplastic B cells can result in growth inhibition and activation-induced cell death (Funakoshi et al., 1994, Blood 83:2787-2794). Thus, CD40 activation in the context of different cell types, transformation, resident TRAFs and co-stimuli can induce responses ranging from activation and proliferation to growth inhibition and apoptosis.

2.2 ANTI-CD40 ANTIBODIES

With at least one exception, the anti-CD40 monoclonal antibodies ("mAbs") described to date are of three general classes: (1) those that block CD40/CD40L interaction by at least 90% and have anti-neoplastic properties (Armitage et al., U.S. Patent No. 5,674,492; Fanslow et al., 1995, Leukocyte Typing V, Schlossman et al., eds., 1:555-556); (2) those that antagonize signaling through CD40 (deBoer et al., U.S. Patent No. 5,677,165); and (3) those that deliver a stimulatory signal through CD40 but do not increase the interaction between CD40 and CD40L, e.g., G28-5, (Ledbetter et al., U.S. Patent No. 5,182,368; PCT Publication WO 96/18413).

One mAb, CD40.4 (5C3) (PharMingen, San Diego, California), has been shown to increase the interaction between CD40 and CD40L by approximately 30-40% (Schlossman et al., eds., 1995, Leukocyte Typing V: White Cell Differentiation Antigens 1:547-556).

Armitage et al. (U.S. Patent No. 5,674,492) describes methods using CD40 binding proteins, including mAb HuCD40-M2, that are capable of binding CD40 and inhibiting the binding of CD40 to CD40L, for preventing or treating disease characterized by neoplastic cells expressing CD40.

DeBoer et al. (U.S. Patent No. 5,677,165) describes anti-CD40 mAbs that, being free of significant agonistic activity, bind to CD40 on the surface of B-cells, and block B-cell activation. An essential feature of U.S. Patent No. 5,677,165 is that upon binding of the anti-CD40 mAb to human CD40 on the surface of normal human B cells, the growth or differentiation of normal human B cells is inhibited.

Ledbetter et al. (U.S. Patent No. 5,182,368) describes a ligand, G28-5, that binds to the B cell surface antigen Bp50 (now designated CD40) and stimulates activated B cells to traverse the cell cycle such that B cell proliferation is augmented. However, G28-5 does not enhance activation of B cells in the presence of CD40L, and does not potentiate CD40/CD40L interaction.

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- S2C6 is an anti-CD40 mAb that was prepared against a human bladder carcinoma (Paulie et al., 1984, Cancer Immunol. Immunother. 17:165-179). S2C6 binds to the CD40 receptor expressed on a variety of cell types including B-lymphocytes, endothelial and epithelial cells. S2C6 has been shown to have specificity toward neoplastic urothelium and B cell-derived malignant lymphocytes. Reactivity with a prostatic carcinoma cell line, HS, and weak reactivity with a melanoma has also been shown (Paulie et al., 1984, Cancer Immunol. Immunother. 17:165-179). Studies have suggested the utility of S2C6 as a diagnostic marker for B cell malignancies (Paulie et al., 1984, Cancer Immunol. Immunother. 17:165-179; Paulie et al., 1985, Eur. J. Cancer. Clin. Oncol. 21:701-710). In addition to detecting B cell malignancies, S2C6 has been shown to deliver strong growth-promoting signals to B lymphocytes (Paulie et al., 1989, J. Immunol. 142:590-595).

- S2C6 has agonistic activity on human peripheral B cells as demonstrated by its ability to stimulate primary B cell proliferation in a dose dependent manner (Paulie et al., 1989, J. Immunol. 142:590-595).

- Although competition studies have shown that G28-5 and S2C6 bind the same or proximal epitopes, the antibodies have been determined to be functionally different based primarily on the stated magnitude of stimulation achieved by either mAb on previously stimulated tonsillar B cells (Clark and Ledbetter, 1986, Proc. Natl. Acad. Sci. USA 83:4494-4498; Ledbetter et al., U.S. Pat. No. 5,182,368). One hundred times more S2C6 compared to G28-5 was required to achieve tonsillar B cell activation under the specific conditions tested (Ledbetter et al., U.S. Patent No. 5,182,368).

- There is a need in the art for therapeutics with increased efficacy to treat or prevent cancer, activate or augment the immune system or treat or prevent an immune deficiency or disorder, a need provided by the present invention.

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Citation or identification of any reference herein shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

Applicants have made the unexpected discovery of a new class of anti-CD40 antibodies that, in addition to delivering a stimulatory signal, enhances the interaction between CD40 and CD40L, enhances CD40L-mediated stimulation and has in vivo anti-neoplastic activity. Production and use of these antibodies and related molecules are facilitated by the inventors' cloning and sequencing of the variable region of mAb S2C6, and identification of the CDR and framework regions therein.

The present invention relates to molecules comprising the variable domain of mAb S2C6 or one or more of the complementarity-determining regions (CDRs) thereof having novel sequences (SEQ ID NO: 3, 4, 8, 9 or 10), which molecules (a) immunospecifically bind CD40 and (b) comprise one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110. In a specific embodiment, the molecules are not native monoclonal antibody S2C6 and do not comprise the native heavy or light chain of said monoclonal antibody S2C6. In a specific embodiment, the molecule is an antibody. In another embodiment, the antibody is not isotype IgG1. In another specific embodiment, the molecule comprises a light chain variable domain, the amino acid sequence of SEQ ID NO:2, or a heavy chain variable domain, the amino acid sequence of SEQ ID NO:7.

The invention further relates to chimeric/fusion proteins comprising a fragment of mAb S2C6 fused to an amino acid sequence of a second protein, as well as to molecules wherein a fragment of mAb S2C6 is covalently bound (e.g., by use of a crosslinking agent) to another chemical structure. In a specific embodiment, a molecule is provided that

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immunospecifically binds CD40, which molecule comprises the heavy and/or light chain variable domain of mAb S2C6 fused to a second protein comprising the amino acid sequence of bryodin 1 (BD1).

- 5 The invention further relates to proteins comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which proteins (a) immunospecifically bind CD40 and (b) comprise one or more
10 substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110. In a specific embodiment, the proteins are not native monoclonal antibody S2C6 and do not comprise the
15 native heavy or light chain of said monoclonal antibody S2C6.

- The invention further relates to purified proteins,
which proteins (a) compete for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-
110, (b) increase the binding of CD40 ligand to CD40 by at
20 least 45%, and (c) comprise one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-
110. In a specific embodiment, the proteins are not native
25 monoclonal antibody S2C6 and do not comprise the native heavy or light chain of said monoclonal antibody S2C6.

- The invention further relates to nucleic acids encoding such molecules and proteins or which hybridize to a DNA consisting of the nucleotide sequence encoding such proteins;
30 recombinant cells comprising such molecules and proteins; and methods of producing such proteins.

- In an embodiment, the isolated nucleic acid comprises a nucleotide sequence encoding a protein comprising (a) a heavy chain variable domain of monoclonal antibody S2C6 as secreted
35 by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and (b) a human constant region.

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In an embodiment, the isolated nucleic acid comprises a nucleotide sequence encoding a protein comprising (a) a light chain variable domain of monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned
5 accession number PTA-110, and (b) a human constant region.

The invention further relates to recombinant cells containing a recombinant nucleic acid vector comprising a nucleotide sequence encoding a protein, which protein competes for binding to CD40 with monoclonal antibody S2C6 as
10 secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%. The invention also provides methods of producing such proteins comprising growing such cells such that the protein
15 is expressed by the cell, and recovering the expressed protein.

The invention further relates to recombinant cells containing a recombinant nucleic acid vector comprising SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID
20 NO:13, SEQ ID NO:14, or SEQ ID NO:15 and methods of producing proteins comprising growing such cells, such that a protein encoded by the nucleotide sequence is expressed by the cell, and recovering the expressed protein.

Pharmaceutical compositions containing the molecules and
25 antibodies of the invention, preferably in purified form, are also provided. In particular embodiments, the invention relates to pharmaceutical compositions comprising a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID
NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which
30 molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as
secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110; and a pharmaceutically
35 acceptable carrier. In a specific embodiment, the molecule is not native monoclonal antibody S2C6 and does not comprise

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the native heavy or light chain of said monoclonal antibody S2C6.

The invention further relates to pharmaceutical compositions comprising a purified protein, which protein (i) competes for binding to CD40 with mAb S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as deposited with the ATCC and assigned accession number PTA-110; and a pharmaceutically acceptable carrier. In a specific embodiment, the protein is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

In specific embodiments, the pharmaceutical compositions of the invention contain the molecules or antibodies of the invention in an amount effective for the treatment or prevention of cancer, or an amount effective for activating or augmenting an immune response, or an amount such that the immune response of the subject is activated or augmented.

In specific embodiments, the pharmaceutical compositions of the invention further comprise CD40 ligand. In a specific embodiment, the pharmaceutical composition comprises in an amount effective for the treatment or prevention of cancer or an immune disorder, or for activating or augmenting an immune response: (a) a molecule that immunospecifically binds CD40, which molecule increases the binding of CD40 ligand to CD40; (b) CD40 ligand; and (c) a pharmaceutically acceptable carrier. In this embodiment, for example, the molecule can be native mAb S2C6 or native mAb 5C3 or an S2C6 derivative as described herein.

The invention further relates to methods for the treatment or prevention of cancer in a subject, for activating or augmenting an immune response in a subject, or for the treatment or prevention of an immune deficiency or disorder in a subject comprising administering to the subject

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a therapeutically effective amount of the molecules or antibodies of the invention, e.g., an amount of a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which
5 molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and
10 assigned accession number PTA-110. In a specific embodiment, the molecule is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

The invention further relates to methods for the
15 treatment or prevention of cancer in a subject, for activating or augmenting an immune response in a subject, or for the treatment or prevention of an immune deficiency or disorder in a subject comprising administering to the subject a purified protein, which protein (i) competes for binding to
20 CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in the primary amino acid
25 sequence relative to native monoclonal antibody S2C6 as deposited with the ATCC and assigned accession number PTA-110. In a specific embodiment, the protein is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

30 In specific embodiments, the methods of the invention further comprise administering CD40 ligand to the subject.

The invention further relates to a method for the treatment or prevention of cancer or an immune disorder in a subject comprising administering to the subject, in an amount effective for said treatment or prevention: (a) a molecule
35 that immunospecifically binds CD40, which molecule increases the binding of CD40 ligand to CD40; and (b) CD40 ligand, in

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which the molecule can be native mAb S2C6 or native mAb 5C3
or any of the S2C6 derivatives described herein.

In a preferred embodiment, the subject is a human.

The invention further relates to a transgenic non-human
5 animal, plant, or an isolated cell containing one or more
transgenes encoding a protein, which protein competes for
binding to CD40 with monoclonal antibody S2C6 as secreted by
the hybridoma deposited with the ATCC and assigned accession
number PTA-110, and which protein increases the binding of
10 CD40 ligand to CD40 by at least 45%.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of the light chain variable region
of S2C6. The nucleotide (SEQ ID NO:1) and amino acid (SEQ ID
15 NO:2) sequences of the light chain variable region ("V_L") are
shown.

Figure 2. Structure of the heavy chain variable region
of S2C6. The nucleotide (SEQ ID NO:6) and amino acid (SEQ ID
NO:7) sequences of the heavy chain variable region ("V_H") of
S2C6 are shown.
20

Figures 3A-3B. Structure of variable regions of S2C6.
(A) The amino acid sequence (SEQ ID NO:2) of S2C6 V_L is shown.
(B) The amino acid sequence (SEQ ID NO:7) of S2C6 V_H is shown.
Complementarity-determining regions ("CDR") are underlined.
The sequences of the four framework regions, adjacent to the
25 CDRs, are shown. The amino acid sequences of V_L CDRs 1-3
correspond to SEQ ID NOS:3-5, respectively. The amino acid
sequences of V_H CDRs 1-3 correspond to SEQ ID NOS:8-10,
respectively.

Figure 4. S2C6 mAb augments CD40-Ig binding to CD40L-
30 expressing Jurkat T cells. CD40-Ig (a soluble fusion protein
of CD40 and human immunoglobulin) binding to surface CD40L
was done in the presence of increasing concentrations of
anti-CD40 monoclonal antibody ("mAb"). mAbs were pre-
incubated for 1 hour with CD40-Ig followed by incubation for
35 1 hour with CD40L-expressing target cells. CD40-Ig binding
to target cells was detected by flow cytometry using a

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fluorescein isothiocyanate ("FITC")-labeled anti-human Ig. The extent of CD40/CD40L binding was then determined from log mean fluorescent intensity ("MFI"). MFI minus background of each population is shown.

5 Figure 5. S2C6 mAb augments binding of soluble CD40L to B cell surface CD40. Ramos B cells, a human B cell lymphoma, were incubated in the presence of increasing concentrations of an anti-CD40 mAb: S2C6, G28-5, or M3 or an irrelevant control mAb, EXA2-1H8. The mAbs were pre-incubated for 1
10 hour with CD40-expressing target cells. Binding of the FITC-labeled CD40L to B cells was then detected directly by flow cytometry. The extent of CD40/CD40L binding was then determined from log mean fluorescent intensity. MFI minus background of each population is shown.

15 Figure 6. S2C6 enhances proliferative response of primary human peripheral B cells in the presence of CD40L* stimulator cells and an anti-CD40 mAb. Peripheral B cells (1×10^5 /well) were combined with increasing numbers of non-proliferative CD40L* Jurkat T stimulator cells and 30 ng/ml of
20 an anti-CD40 mAb: S2C6, G28-5, or M3 or the control mAb, EXA2-1H8. B cell proliferation was measured by ^3H -TdR incorporation at 72 h following addition of stimulus.

25 Figure 7. Comparative proliferative response of primary human peripheral B cells to an anti-CD40 mAb in the presence or absence of CD40L. Peripheral B cells were combined with non-proliferative CD40L* stimulator cells at a fixed ratio of 4:1 and increasing concentrations of an anti-CD40 mAb: S2C6 G28-5 or the control antibody, EXA2-1H8. B cell
30 proliferation was measured by ^3H -TdR incorporation at 72 h following addition of stimulus.

35 Figures 8A-8C. Anti-tumor activity of mAb S2C6 *in vivo*. Anti-tumor activity of S2C6 against (A) Ramos human B cell non-Hodgkin's lymphoma, (B) HS Sultan multiple myeloma, or (C) IM-9 multiple myeloma was assessed. SCID mice (5/group) were pretreated or not with anti-asialo-GM1 to inhibit natural killer ("NK") activity and treated with mAb on day 1

or day 5 following injection of 1×10^6 - 2×10^6 tumor cells.
Solid lines indicate the number of surviving mice over time.

Figure 9. BD1-S2C6 sFv specifically binds to immobilized CD40-Ig in ELISA. BD1-S2C6 sFv (single-chain anti-CD40 immunotoxin consisting of bryodin 1 (BD1) fused to the variable region of monoclonal antibody S2C6) was expressed in *E. coli* as inclusion bodies, denatured and refolded. The refolded protein was then isolated using Blue Sepharose followed by affinity chromatography over immobilized CD40-Ig. The purified protein was then tested for binding to immobilized CD40-Ig in ELISA. Microtiter plates were coated with CD40-Ig at 0.5 $\mu\text{g/ml}$ followed by the addition of dilutions of purified BD1-S2C6 sFv in the presence of 25 $\mu\text{g/ml}$ S2C6 mAb (\blacktriangle), 25 $\mu\text{g/ml}$ control antibody BR96 (\bullet), or no excess antibody (\blacksquare). Binding of BD1-S2C6 sFv to the immobilized receptor was detected by the addition of BD1-specific rabbit antiserum followed by the addition of horseradish peroxidase conjugated goat anti-rabbit Ig. The binding of BD1-S2C6 sFv to CD40-Ig was completely inhibited by the addition of excess S2C6 mAb but not by the addition of the control mAb.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to proteins encoded by and nucleotide sequences of S2C6 genes. The invention further
25 relates to fragments and other derivatives and analogs of such S2C6 proteins and nucleic acids. In various specific embodiments, the molecules (e.g., antibodies) of the invention comprise all or a portion of mAb S2C6 (the light chain and/or heavy chain, or light chain CDR 1 (SEQ ID NO:3)
30 and/or 2 (SEQ ID NO:4), and/or heavy chain CDR 1 (SEQ ID NO:8), 2 (SEQ ID NO:9), and/or 3 (SEQ ID NO:10), or light chain CDR3 (SEQ ID NO:5) in combination with any of the other CDRs and/or one or more of the four heavy chain and four light chain framework regions, provided that such molecules
35 are not native mAb S2C6 as deposited with the ATCC and assigned accession number PTA-110 or the heavy or light chain thereof. Such molecules may differ from S2C6 in sequence

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- and/or in post-translational modification (glycosylation, amidation, peptide bonding or cross-linking to a non-S2C6 sequence, etc.). In various specific embodiments, a molecule of the invention immunospecifically binds CD40 (or when multimerized immunospecifically binds CD40), competes with native S2C6 for binding to CD40, and/or increases the binding of CD40 ligand to CD40 by at least 45%, 50%, 60% or 65%. Nucleic acids encoding such molecules, e.g., S2C6 fragments or derivatives, are also within the scope of the invention, as well as nucleic acids encoding native mAb S2C6.
- 10 Production of the foregoing proteins, e.g., by recombinant methods, is provided.

- The invention also relates to S2C6 proteins and derivatives including but not limited to fusion/chimeric proteins which are functionally active, i.e., which are capable of displaying one or more known functional activities associated with a full-length S2C6 mAb. Such functional activities include but are not limited to ability to bind CD40, delivery of a stimulatory signal to the CD40 signaling pathway (e.g., so as to cause B cell proliferation), potentiation of the interaction of CD40L with CD40; ability to inhibit tumor growth; and ability to induce an immune response.

- Antibodies to CD40 comprising S2C6, its derivatives and analogs including but not limited to humanized antibodies, single chain antibodies; bispecific antibodies; and antibodies conjugated to chemotherapeutic agents or biological response modifiers, are additionally provided.

- The invention further relates to methods of treating or preventing cancer, inflammatory diseases and disorders of the immune system comprising administering a composition of the invention alone or in combination with CD40L.

- The invention is illustrated by way of examples set forth in Sections 6-9 below which disclose, *inter alia*, the cloning and characterization of S2C6 genes; the potentiation of the CD40/CD40L interaction; inhibition of tumor growth; and binding of a single-chain anti-CD40 immunotoxin to CD40-Ig.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5.1 ISOLATION OF S2C6 GENES

5 The invention relates to the nucleotide sequences of S2C6 nucleic acids. In specific embodiments, S2C6 nucleic acids comprise the cDNA sequences of SEQ ID NOS:1 and 6, or nucleic acids encoding an S2C6 protein (e.g., a protein having the sequence of SEQ ID NOS:2 and 7). The invention
10 provides purified nucleic acids consisting of at least 8 nucleotides (i.e., a hybridizable portion) of an S2C6 gene sequence; in other embodiments, the nucleic acids consist of at least 25 (contiguous) nucleotides, 50 nucleotides, 100, or 200 nucleotides of an S2C6 sequence, or a full-length S2C6
15 variable region coding sequence. In the same or other embodiments, the nucleic acids are smaller than 50, 75, 100, or 200 or 5000 nucleotides in length. Nucleic acids can be single or double stranded. The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences or their reverse complements, and in
20 particular, such nucleic acids that encode proteins that bind to CD40, compete with S2C6 for binding to CD40, and/or increase the binding of CD40 ligand to CD40 by at least 45%, 50%, 60%, or 65%. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least
25 10, 25, 50, 100, or 200 nucleotides or the entire coding region of an S2C6 variable region gene.

Nucleic acids encoding derivatives and analogs of S2C6 proteins are additionally provided. As is readily apparent, as used herein, a "nucleic acid encoding a fragment or
30 portion of an S2C6 protein" shall be construed as referring to a nucleic acid encoding only the recited fragment or portion of the S2C6 protein and not the other contiguous portions of the S2C6 protein as a continuous sequence.

5.2 CLONING PROCEDURES

35 Specific embodiments for the cloning of an S2C6 gene follow. In a specific embodiment, total RNA is isolated from

a mAb S2C6-producing hybridoma and polymerase chain reaction is used to amplify desired variable region sequences, using primers based on the sequences disclosed herein. For an illustrative example, see Section 6, *infra*. By way of
5 another example, mRNA is isolated from a mAb S2C6-producing hybridoma, cDNA is made and ligated into an expression vector (e.g., a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to
10 select for the expressed product. In one embodiment, selection is on the basis of hybridization to a labeled probe representing a portion of an S2C6 gene or its RNA or a fragment thereof (Benton and Davis, 1977, *Science* 196:180; Grunstein and Hogness, 1975, *Proc. Natl. Acad. Sci. U.S.A.*
15 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available.
20 Further selection can be carried out on the basis of the properties of the gene.

Alternatively, the presence of the desired gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For
25 example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected and expressed to produce a protein that has, e.g., similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, or functional activity, as known for an S2C6
30 protein. For example, ability to bind CD40 can be detected in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

An S2C6 gene can also be identified by mRNA selection using nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to
35 isolate complementary mRNAs by hybridization. Functional assays (e.g., binding to CD40, etc.) of the *in vitro*

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translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences.

In another embodiment, the S2C6 cDNA can be chemically synthesized from the sequence disclosed herein. Other methods of isolating S2C6 genes known to the skilled artisan can be employed.

The identified and isolated S2C6 gene/cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and an S2C6 gene may be modified by homopolymeric tailing, or by PCR with primers containing the appropriate sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated S2C6 gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene
 5 may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The S2C6 sequences provided by the instant invention
 10 include those nucleotide sequences encoding substantially the same amino acid sequences as found in native S2C6 variable regions, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other S2C6 derivatives or analogs, as described
 15 below for S2C6 derivatives and analogs.

5.3 EXPRESSION OF S2C6 GENES

The nucleotide sequence coding for an S2C6 protein or a functionally active analog or fragment or other derivative thereof (see Section 5.6), can be inserted into an
 20 appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native S2C6 gene and/or its flanking regions.
 25 A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such
 30 as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA; transgenic plants or transgenic non-human animals. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized,
 35 any one of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of a nucleic acid sequence encoding an S2C6 protein or peptide fragment may be regulated by a second nucleic acid sequence so that the S2C6 protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of an S2C6 protein may be controlled by any promoter/enhancer element known in the art. Promoters that are not native S2C6 gene promoters which may be used to control S2C6 gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the lac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following

- animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); a gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), an immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogham et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to an S2C6 gene nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (e.g., an antibiotic resistance gene).

Expression vectors containing S2C6 gene inserts can be identified by three general approaches: (a) nucleic acid hybridization; (b) presence or absence of "marker" gene

functions; and (c) expression of inserted sequences. In the first approach, the presence of an S2C6 gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted S2C6 gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of an S2C6 gene in the vector. For example, if the S2C6 gene is inserted within the marker gene sequence of the vector, recombinants containing the S2C6 insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the S2C6 product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the S2C6 protein in *in vitro* assay systems, e.g., potentiation of CD40L binding with CD40; stimulation of proliferation of normal B cells; inhibition of tumor growth.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda phage), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus,

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expression of the genetically engineered S2C6 protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a non-glycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

In specific embodiments, the S2C6-related protein that is expressed is an antibody or fragment or derivative thereof. The recombinant antibody may contain a recombinant light chain variable domain, a recombinant heavy chain variable domain, or both. In a specific embodiment, both light and heavy chains or derivatives thereof are recombinantly expressed by a cell (see e.g., U.S. Patent No. 4,816,397 dated March 28, 1989 by Boss et al.) A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA; transgenic plants or transgenic non-human animals.

5.4 IDENTIFICATION AND PURIFICATION OF GENE PRODUCTS

In particular aspects, the invention provides amino acid sequences of S2C6 proteins and fragments and derivatives thereof which comprise a complementarity-determining region (CDR) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" S2C6 material as used herein refers to that material

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displaying one or more functional activities associated with a full-length (native) S2C6 protein, e.g., binding to CD40; stimulation of proliferation of normal B cells; inhibition of tumor growth; increase the binding of CD40 ligand to CD40 by at least 45%.

5

In specific embodiments, the invention provides fragments of an S2C6 protein consisting of at least 6 amino acids, 10 amino acids, 20 amino acids, 50 amino acids, 75 amino acids or of at least 100 amino acids and nucleic acids encoding the foregoing.

10

Once a recombinant which expresses the S2C6 gene sequence is identified, the gene product can be analyzed.

This is achieved by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel

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electrophoresis, immunoassay; stimulation of proliferation of normal B cells; CD40 binding assays, promotion of the binding of CD40 ligand to CD40, inhibition of tumor growth, etc.

Once the S2C6 protein is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column

20

chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay (see Section 5.7).

Alternatively, the S2C6 protein or derivative thereof can be synthesized by standard chemical methods known in the art based on the sequence disclosed herein (e.g., see Hunkapiller et al., 1984, Nature 310:105-111).

25

In a specific embodiment of the present invention, such S2C6 proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid sequence substantially as depicted in Figures 3A-3B (SEQ ID NOS:2 and 7), as well as fragments and other derivatives, and analogs thereof, including proteins

30

35 homologous thereto.

5.5 STRUCTURE OF S2C6 GENES AND PROTEINS

The structure of S2C6 genes and proteins of the invention can be analyzed by various methods known in the art. Some examples of such methods are described below.

5

5.5.1 GENETIC ANALYSIS

The cloned DNA or cDNA corresponding to an S2C6 gene can be analyzed by methods including but not limited to Southern hybridization (Southern, 1975, J. Mol. Biol. 98:503-517), Northern hybridization (see e.g., Freeman et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4094-4098), restriction endonuclease mapping (Maniatis, 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), and DNA sequence analysis. Accordingly, this invention provides nucleic acid probes recognizing an S2C6 gene. For example, polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7652-7656; Ochman et al., 1988, Genetics 120:621-623; Loh et al., 1989, Science 243:217-220) followed by Southern hybridization with an S2C6 gene-specific probe can allow the detection of an S2C6 gene in DNA or cDNA from a cell (e.g., hybridoma). Methods of amplification other than PCR are commonly known and can also be employed. The stringency of the hybridization conditions for both Southern and Northern hybridization can be manipulated to ensure detection of nucleic acids with the desired degree of relatedness to the specific S2C6 gene probe used. Modifications of these methods and other methods commonly known in the art can be used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of an S2C6 gene. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), the use of T7 DNA polymerase (Tabor and

Richardson, U.S. Patent No. 4,795,699), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, California).

5.5.2 PROTEIN ANALYSIS

The amino acid sequence of an S2C6 protein can be derived by deduction from the DNA sequence, or alternatively, by direct sequencing of the protein, e.g., with an automated amino acid sequencer.

An S2C6 protein sequence can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic (potentially immunogenic) regions of the S2C6 protein and the corresponding regions of the gene sequence which encode such regions.

Secondary, structural analysis (Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of an S2C6 protein that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, open reading frame prediction and plotting, as well as determination of sequence homologies, can also be accomplished using computer software programs available in the art.

5.6 mAb S2C6 ANTIBODY DERIVATIVES

Described herein are methods for the production of S2C6 antibody derivatives capable of immunospecifically binding CD40.

Such antibodies include but are not limited to monoclonal, humanized, chimeric, single chain, bispecific, Fab fragments, F(ab'), fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

For preparation of additional monoclonal antibodies to CD40, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256, 495-497;

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and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kozbor *et al.*, 1983, *Immunology Today* 4, 72; Cole *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80, 2026-2030), and the EBV-hybridoma technique to produce human monoclonal
5 antibodies (Cole *et al.*, 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies or other anti-CD40 antibodies available in the art may, e.g., be used as the basis from which to clone and thus supply a complementary light chain if a S2C6 heavy chain is
10 to be recombinantly expressed (the two chains may be recombinantly expressed in the same cell or combined *in vitro* after separate expression and purification); alternatively, a light chain from an antibody of any specificity may be used. Nucleic acids (e.g., a plasmid) encoding a S2C6 heavy chain
15 or encoding a molecule comprising a S2C6 heavy chain variable domain can be transfected into a cell expressing an antibody light chain or molecule comprising an antibody light chain, for expression of a multimeric protein; the antibody light chain can be recombinant or non-recombinant, and may or may
20 not have anti-CD40 specificity. Alternatively, S2C6 heavy chains or molecules comprising the variable region thereof or a CDR thereof can optionally be expressed and used without the presence of a complementary light chain or light chain variable region. In various embodiments, the invention
25 provides a S2C6 heavy chain with CD40 binding affinity, or a molecule consisting of or (alternatively) comprising one or more copies of heavy chain CDR 8, 9, and/or 10, or a protein (peptide or polypeptide) the sequence of which consists of, or comprises, one or more copies of CDR 8, 9 or 10. In a specific embodiment, such a protein can be N or C-terminal
30 modified, e.g., by C-terminal amidation or N-terminal acetylation.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison, *et al.*, 1984, *Proc. Natl. Acad. Sci.*, 81, 6851-6855; Neuberger, *et al.*, 1984, *Nature*
35 312, 604-608; Takeda, *et al.*, 1985, *Nature* 314, 452-454) by splicing the genes from a mouse antibody molecule of

derivative thereof immunospecifically binds CD40. Preferably, the set of framework regions is from a human monoclonal antibody, e.g., a human monoclonal antibody that does not bind CD40.

5 In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a light chain variable domain, said variable domain comprising (a) a set of three complementarity-determining regions (CDRs), in which said set of CDRs comprises SEQ ID NO:3 or SEQ ID NO:4, and
10 (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in the light chain of monoclonal antibody S2C6, and in which said antibody or derivative thereof immunospecifically binds CD40.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242, 423-426; Huston, et al., 1988, Proc. Natl. Acad. Sci. USA 85, 5879-5883; and Ward, et al., 1989, Nature 334, 544-546) can be adapted to produce single chain antibodies using S2C6 sequences. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. In a specific embodiment, the single chain antibody comprises the amino acid sequences as depicted in Figure 3A and 3B (SEQ ID NOS:2 and 7, respectively).

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In particular aspects, the proteins, derivatives, or analogs are encoded by the sequence of SEQ ID NO:1 or SEQ ID NO:6.

The production and use of derivatives and analogs related to an S2C6 protein are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length, S2C6 protein. As one example, such derivatives or analogs which have the desired binding specificity can be used in immunoassays, or therapeutically for inhibition of tumor growth, etc. A specific embodiment relates to an S2C6 protein fragment that binds CD40 and potentiates binding of CD40L to CD40. Derivatives or analogs of an S2C6 protein can be tested for the desired activity by various immunoassays known in the art, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, Western blots, immunofluorescence assays, protein A assays, immunoelectrophoretic assays, etc.

In addition, assays known in the art can be used to detect or measure the ability to inhibit cell proliferation (e.g., inhibition of tumor cell growth) or ability to stimulate cell proliferation (e.g., proliferation of B cells) *in vivo* or *in vitro*.

In particular, S2C6 derivatives can be made by altering S2C6 sequences by substitutions, additions (e.g., insertions) or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an S2C6 gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of an S2C6 gene which is altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the S2C6 derivatives of the invention include, but are not limited to, those containing, as a

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primary amino acid sequence, all or part of the amino acid sequence of an S2C6 protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a
5 silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutions for an amino acid within the sequence may be selected from other members
10 of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine,
15 asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such substitutions are generally understood to be conservative substitutions.

20 In a specific embodiment of the invention, proteins consisting of or comprising a fragment of an S2C6 protein consisting of at least 10 (continuous) amino acids of the S2C6 protein is provided. In other embodiments, the fragment consists of at least 20 or at least 50 amino acids of the S2C6 protein. In specific embodiments, such fragments are
25 not larger than 50, 75, 100, or 200 amino acids. Derivatives or analogs of S2C6 proteins include but are not limited to those molecules comprising regions that are substantially homologous to an S2C6 protein or fragment thereof (e.g., in various embodiments, at least 60% or 70% or 80% or 90% or 95%
30 identity over an amino acid sequence of identical size with no insertions or deletions or when compared to an aligned sequence in which the alignment is done by a computer ~~homology program known in the art~~) or whose encoding nucleic acid is capable of hybridizing to a coding S2C6 gene
35 sequence, under high stringency, moderate stringency, or low stringency conditions.

Specifically, by way of example computer programs for determining homology may include but are not limited to TBLASTN, BLASTP, FASTA, TEASTA, and CLUSTALW (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85(8):2444-8; 5 Altschul et al., 1990, J. Mol. Biol. 215(3):403-10; Thompson, et al., 1994, Nucleic Acids Res. 22(22):4673-80; Higgins, et al., 1996, Methods Enzymol 266:383-402; Altschul, et al., 1990, J. Mol. Biol. 215(3):403-10). Default parameters for each of these computer programs are well known and can be

utilized.

Specifically, Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) (Altschul et al., 1990, J. of Molec. Biol., 215:403-410, "The BLAST Algorithm; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402) is a heuristic search 15 algorithm tailored to searching for sequence similarity which ascribes significance using the statistical methods of Karlin and Altschul 1990, Proc. Natl Acad. Sci. USA, 87:2264-68; 1993, Proc. Nat'l Acad. Sci. USA 90:5873-77. Five specific BLAST programs perform the following tasks: 1) The BLASTP program compares an amino acid query sequence against a 20 protein sequence database; 2) The BLASTN program compares a nucleotide query sequence against a nucleotide sequence database; 3) The BLASTX program compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database; 25 4) The TBLASTN program compares a protein query sequence against a nucleotide sequence database translated in all six reading frames (both strands); 5) The TBLASTX program compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide 30 sequence database.

Smith-Waterman (database: European Bioinformatics Institute www.ebi.ac.uk/bic_sw/) (Smith-Waterman, 1981, J. of Molec. Biol., 147:195-197) is a mathematically rigorous algorithm for sequence alignments. 35 FASTA (see Pearson et al., 1988, Proc. Nat'l Acad. Sci. USA, 85:2444-2448) is a heuristic approximation to the Smith-

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Waterman algorithm. For a general discussion of the procedure and benefits of the BLAST, Smith-Waterman and FASTA algorithms see Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods"

5 (www.psc.edu) and references cited therein.

The S2C6 derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned S2C6 gene
10 sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction
15 endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a modified gene encoding a derivative or analog of the S2C6 protein, care should be taken to ensure that the modified gene remains within the same translational reading
20 frame as the native protein, uninterrupted by translational stop signals, in the gene region where the desired S2C6 protein activity is encoded.

Additionally, an S2C6 nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy
25 translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed
30 mutagenesis (Hutchinson et al., 1978, *J. Biol. Chem.* 253:6551), PCR with primers containing a mutation, etc.

Manipulations of an S2C6 protein sequence may also be made at the protein level. Included within the scope of the invention are S2C6 protein fragments or other derivatives or
35 analogs which are differentially modified during or after translation, e.g., by glycosylation, acetylation,

phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH_4 , acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

10 In addition, analogs and derivatives of an S2C6 protein can be chemically synthesized. For example, a peptide corresponding to a portion of an S2C6 protein which comprises the desired domain, or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer.

15 Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the S2C6 sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, tbutyglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, C α -methyl amino acids, N α -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

25

In other specific embodiments, the S2C6 protein, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence of a different protein). The heterologous protein sequence can comprise a biological response modifier, including but not limited to interferon- α , 35 interferon γ , interleukin-2, interleukin-4, interleukin-6, and tumor necrosis factor, or a functionally active portion

- thereof. Alternatively, the heterologous protein sequence can comprise enzymes such as β -lactamase or carboxylesterases or toxins such as bryodin 1, *Pseudomonas* exotoxin A, or gelonin, or a functionally active portion thereof.
- 5 Additionally, the S2C6 protein can be chemically linked to chemotherapeutic agents, including but not limited to alkylating agents (e.g. nitrogen mustards, nitrosoureas, triazenes); antimetabolites (e.g. folic acid analogs, pyrimidine analogs, purine analogs); natural products (e.g. antibiotics, enzymes, biological response modifiers);
- 10 miscellaneous agents (e.g. substituted urea, platinum coordination complexes); and hormones and antagonists (e.g. estrogens, androgens, antiandrogen, gonadotropin releasing hormone analog); or functionally active portion thereof (see, e.g., Goodman and Gilman, *The Pharmacological Basis of*
- 15 *Therapeutics*, Ninth Edition, McGraw-Hill, pp. 1225-1287, 1996). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a
- 20 chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. In different embodiments, the heterologous protein sequence can be covalently bound to the S2C6-related sequences by other than a peptide bond, e.g., by use of chemical crosslinking agents
- 25 well known in the art.

In a specific embodiment, an S2C6 protein derivative is a chimeric or fusion protein comprising an S2C6 protein or fragment thereof (preferably consisting of at least a domain or motif of the S2C6 protein, or at least 10, 50 or 100 amino

30 acids of the S2C6 protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In a specific embodiment, the different protein is a toxin, enzyme or biological response modifier.

In specific embodiments, the amino acid sequence of the

35 different protein is at least 6, 10, 20 or 30 continuous amino acids of the different protein or a portion of the

different protein that is functionally active. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising an S2C6-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. Chimeric genes comprising portions of an S2C6 gene fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment of an S2C6 protein of at least 6 or 15 or 50 amino acids, or a fragment that displays one or more functional activities of the S2C6 protein (e.g., comprising copies of one or more CDRs).

In a specific embodiment, the S2C6 protein or derivative thereof is chemically linked to a chemotherapeutic drug including but not limited to doxorubicin, paclitaxel or docetaxel. Such a S2C6-drug conjugate can deliver the drug to cells expressing CD40. One or more drug molecules can be linked to the S2C6 protein or derivative. Linkages include but are not limited to hydrazone, peptide or carbohydrate linkages.

In another specific embodiment, the derivative is a molecule comprising a region of homology with an S2C6 protein. By way of example, in various embodiments, a first protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region (without any insertions or deletions) or when compared to an aligned sequence of the

second region that has been aligned by a computer homology program known in the art.

5.8 HYBRIDIZATION CONDITIONS

5 In a specific embodiment, a nucleic acid which is hybridizable to an S2C6 nucleic acid (e.g., having a sequence as set forth in SEQ ID NOS:1 or 6), or to its reverse complement, or to a nucleic acid encoding an S2C6 derivative, or to its reverse complement under conditions of low stringency is provided. By way of example and not
10 limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78, 6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5),
15 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10⁶ cpm ³²P-labeled probe
20 is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If
25 necessary, filters are washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency which may be used are well known in the art (e.g., as employed for cross-species hybridizations).

In a specific embodiment, a nucleic acid which is
30 hybridizable to an S2C6 nucleic acid (e.g., having a sequence as set forth in SEQ ID NOS:1 or 6), or to its reverse complement, or to a nucleic acid encoding an S2C6 derivative, or to its reverse complement under conditions of high stringency is provided. By way of example and not
35 limitation, procedures using such conditions of high stringency are as follows. Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in

- buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μ g/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 μ g/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.
- 10 In a specific embodiment, a nucleic acid which is hybridizable to an S2C6 nucleic acid (e.g., having a sequence as set forth in SEQ ID NOS:1 or 6), or to its reverse complement, or to a nucleic acid encoding an S2C6 derivative, or to its reverse complement under conditions of moderate stringency is provided. Selection of appropriate conditions for such stringencies is well known in the art (see e.g., Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; see also, Ausubel et al., eds., in the Current Protocols in Molecular Biology series of laboratory technique manuals, 1987-1997 Current Protocols,[®] 1994-1997 John Wiley and Sons, Inc.).

5.9 THERAPEUTIC USES

- The invention provides for treatment or prevention of various diseases or disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such Therapeutics include but are not limited to: S2C6 antibodies and derivatives thereof (e.g., as described hereinabove); and nucleic acids encoding such S2C6 antibodies and derivatives (e.g., as described hereinabove). "Treatment" as used herein shall be deemed to include any clinically desirable or beneficial effect on the disease or disorder, including but not limited to alleviation of one or more symptoms, regression, slowing or cessation of progression, etc.
- 35 In specific embodiments of the invention, the Therapeutic is administered alone or in combination with

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CD40L for the treatment or prevention of malignancies (including but not limited to carcinoma and hematologic malignancies), inflammatory diseases, and disorders of the immune system. The Therapeutic and CD40L can, but need not be, contained within the same formulation, i.e., administration of the Therapeutic and CD40 can be performed separately but concurrently or during the same course of treatment. In a specific embodiment, the malignant cells express CD40. Alternatively, the cells of the malignancy need not express CD40, since endothelial cells of the vasculature associated with a malignant tumor should express CD40 and thus the Therapeutic of the invention should provide treatment efficacy even for tumors that do not express CD40. In a preferred embodiment, the Therapeutic potentiates the binding of CD40L to CD40 by at least 45%, 50%, 60%, or 65%.

In specific embodiments, the Therapeutic is used to increase the immune response of an immunosuppressed individual, such as a person suffering from acquired immunodeficiency syndrome, from malignancy, or an infant or elderly person.

In other embodiments of the invention, the Therapeutic may be chemically modified so that cells that it binds to are killed. Such cells include but are not limited to multiple myeloma cells, lymphoma cells or carcinomas. Since all B-cells express CD40, this approach can result in suppression of the immune response. For example, a cytotoxic drug linked to S2C6 sequences (e.g., a fusion protein) may be used in vivo to cause immunosuppression in order to cross histocompatibility barriers in transplant patients; alternatively, these modified ligands may be used to control autoimmune diseases.

In other embodiments, the Therapeutic may be used to promote the proliferation and/or differentiation of CD40-bearing cells that are not B cells, for example, lung carcinoma cells, as a means of directly treating malignancy or as an adjunct to chemotherapy.

Malignancies which may be treated or prevented using a
Therapeutic of the invention include but are not limited to
those in Table 1:

5

TABLE 1
MALIGNANCIES AND RELATED DISORDERS

	Leukemia
	acute leukemia
	acute lymphocytic leukemia
10	acute myelocytic leukemia
	myeloblastic
	promyelocytic
	myelomonocytic
	monocytic
	erythroleukemia
	chronic leukemia
	chronic myelocytic (granulocytic) leukemia
	chronic lymphocytic leukemia
15	Polycythemia vera
	Lymphoma
	Hodgkin's disease
	non-Hodgkin's disease
	Multiple myeloma
	Waldenström's macroglobulinemia
	Heavy chain disease
	Solid tumors
20	sarcomas and carcinomas
	fibrosarcoma
	myxosarcoma
	liposarcoma
	chondrosarcoma
	osteogenic sarcoma
	osteosarcoma
	chordoma
25	angiosarcoma
	endotheliosarcoma
	lymphangiosarcoma
	lymphangioendotheliosarcoma
	synovioma
	mesothelioma
	Ewing's tumor
	leiomyosarcoma
30	rhabdomyosarcoma
	colon carcinoma
	colorectal carcinoma
	pancreatic cancer
	breast cancer
	ovarian cancer
	prostate cancer
	squamous cell carcinoma
35	basal cell carcinoma
	adenocarcinoma
	sweat gland carcinoma
	sebaceous gland carcinoma

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5 papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
10 testicular tumor
lung carcinoma
small cell lung carcinoma
non small cell lung carcinoma
bladder carcinoma
epithelial carcinoma
glioma
15 astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
menangioma
20 melanoma
neuroblastoma
retinoblastoma
nasopharyngeal carcinoma
esophageal carcinoma

25 Inflammatory diseases and deficiencies or disorders of
the immune system which may be treated or prevented using a
Therapeutic of the invention include but are not limited to
those in Table 2:

30

TABLE 2
INFLAMMATORY DISEASES AND
IMMUNE SYSTEM DISORDERS

35 systemic lupus erythematosus (SLE)
Scleroderma (e.g., CRST syndrome)
inflammatory myositis
Sjögren's syndrome (SS)

- mixed connective tissue disease (e.g., MCTD, Sharp's syndrome)
rheumatoid arthritis
multiple sclerosis
inflammatory bowel disease (e.g., ulcerative colitis, Crohn's disease)
5 acute respiratory distress syndrome
pulmonary inflammation
osteoporosis
delayed type hypersensitivity
asthma
primary biliary cirrhosis (PBC)
idiopathic thrombocytopenic purpura (ITP)

10

5.9.1 EFFECTIVE DOSE

Toxicity and therapeutic efficacy of such Therapeutics can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Therapeutics that exhibit large therapeutic indices are preferred. While Therapeutics that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. Exemplary doses include but are not limited to from 1 ng/kg to 100 mg/kg. The dosage of such Therapeutics lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For a Therapeutic, the therapeutically effective dose may preferably be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the

IC₅₀ (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma
5 may be measured, for example, by high performance liquid chromatography.

5.9.2 FORMULATIONS

Pharmaceutical compositions for use in accordance with
10 the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the Therapeutics and their physiologically acceptable salts and solvates may be formulated for
15 administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules
20 prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate)
25 lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form
30 of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending
35 agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily

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esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

- 5 Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

- 10 For administration by inhalation, the Therapeutics for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane,
15 trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be
20 formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

- The Therapeutics may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be
25 presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with or without an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain
formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may
30 be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

- The Therapeutics may also be formulated in rectal compositions such as suppositories or retention enemas, e.g.,
35 containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the Therapeutics may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the Therapeutics may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration preferably for administration to a human.

In specific embodiments, the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a Therapeutic in combination with CD40 ligand.

The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

6. EXAMPLE: CLONING OF SC26 VARIABLE REGIONS

6.1 MATERIALS AND METHODS

The S2C6 light chain and heavy chain variable regions were cloned using methods essentially as described in Gilliland et al., 1996, Tissue Antigens 47:1-20. Total RNA was isolated from the S2C6 hybridoma. First strand complementary DNA (cDNA) was prepared for the mouse kappa light chain and heavy chain variable regions using reverse transcriptase and anti-sense primers that annealed approximately 100 base pairs downstream of the JC junction.

A poly-G tail was added to the cDNA strands using terminal transferase and then double stranded DNA was synthesized using the polymerase chain reaction (PCR). The PCR primers, specific for the poly-G tail or a sequence approximately 50 bases inside the cDNA for the light chain or heavy chain, were designed to include unique restriction sites. After amplification, the PCR products were digested with *EcoRI* and *HindIII* cloned into pUC19 that had been digested with the same restriction enzymes. These reactions were ligated, transformed into *E. coli* DH5 α , and the resulting clones were screened by restriction analysis. Clones that were positive by restriction digestion analysis were sequenced by DNA sequencing on a Li-Cor fluorescence sequencer. The nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of the light chain variable region (V_L) are shown in Figure 1. The nucleotide (SEQ ID NO:6) and amino acid (SEQ ID NO:7) sequences of the heavy chain variable region (V_H) are shown in Figure 2. Figures 3A-3B illustrate the amino acid sequence of S2C6 V_L and S2C6 V_H (Figure 3A and 3B, respectively). The CDRs are underlined. The amino acid sequences of V_L CDRs 1-3 correspond to SEQ ID NOS:3-5, respectively. The amino acid sequences of V_H CDRs 1-3 correspond to SEQ ID NOS:8-10, respectively.

The resulting DNA sequences were then compared to the light chain and heavy chain variable regions of other murine antibodies of the same isotype and the reading frame and corresponding amino acid sequences for the genes isolated from S2C6 were determined. To confirm the amino acid sequences, the light chain and heavy chain variable regions of S2C6 mAb were subjected to N-terminal amino acid analysis.

The amino acid sequences of S2C6 V_L , S2C6 V_H and the CDRs of both the V_L and V_H were submitted for BLASTP searches on April 21, 1999 using both the NR database (All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF) and the Kabat database (Kabat's database of sequences of immunological interest). The sequences found using the NR database can be retrieved using the Accession number at

http://www.ncbi.nlm.nih.gov. The sequences found using the Kabat database can be retrieved using the Accession number at http://immuno.bme.nwu.edu/database_.html and SEQHUNT II. The results of these searches are shown below:

5

BLASTP SEARCHES USING NR DATABASE

S2C6 VL (SEQ ID NO:2): a BLASTP search of the NR database with S2C6 VL as the query yielded no hits with 100% identity and 6 hits with 94% (106/112) identity. These 6 are shown below:

15 pir||PT0359 IgG kappa chain V region (R4A.12) - mouse (fragment)
gi|196660 (M59949) immunoglobulin kappa-chain VJ region [Mus musculus]
gi|196954 (M12183) kappa-chain V-region [Mus musculus]>gi|2247 [Mus musculus]
pir||B34904 Ig kappa chain precursor V region (12-40 and 5-14) ...
20 emb|CAA80076| (Z22102) immunoglobulin variable region [Mus musculus]
dbj|BAA22172| (AB006833) anti-pseudouridine monoclonal antibody...

25 VL CDR1 (SEQ ID NO:3): a BLASTP search with VL CDR1 as the query yielded no hits with 100% identity and numerous hits with 93% identity (15/16). The first 5 of these are shown below:

30 dbj|BAA03480| (D14627) immunoglobulin gamma-3 kappa chain [Mus musculus]
dbj|BAA22172| (AB006833) anti-pseudouridine monoclonal antibody...
gi|4101647 (AF005352) immunoglobulin V-region light chain [Mus musculus]
gi|3377681 (AF078800) single chain anti-HIV-1 Rev
35 variable fragment...

gi|1870366 (U55625) anti-DNA immunoglobulin light chain
IgM [Mus musculus]

VL CDR2 (SEQ ID NO:4): a BLASTP search of the NR database
5 with VL CDR2 as the query yielded no hits.

VL CDR3 (SEQ ID NO:5): a BLASTP search of the NR database
with VL CDR3 as the query yielded no hits.

10 S2C6 VH (SEQ ID NO:7): a BLASTP search of the NR database
using S2C6 VH as a query yielded no hits with 100% identity
and numerous hits with up to 88% identity the first 5 of
which are shown below:

gi|3561044 (AF083186) anti-HIV-1 p24 antibody D2 heavy
15 chain [Mus musculus]

pdb|1A6T|B Chain B, Fab Fragment Of Mab1-1a Monoclonal
Antibody

gi|2895955 (AF045895) IgG1 heavy chain mAB1-1A [Mus
musculus]

20 emb|CAA80023| (Z22049) immunoglobulin variable region
[Mus musculus]

gi|194510 (M91695) immunoglobulin gamma-1 chain [Mus
musculus]

25 VH CDR1 (SEQ ID NO:8): a BLASTP search of the NR database
with VH CDR1 as the query yielded no hits.

VH CDR2 (SEQ ID NO:9): a BLASTP search of the NR database
with VH CDR2 as the query yielded no hits with 100% identity,
1 hit with 94% identity (16/17) and numerous hits with less
30 than 94% identity. The 1 hit with 94% identity is shown:

gi|3561044 (AF083186) anti-HIV-1 p24 antibody D2 heavy
chain [Mus musculus]

35 VH CDR3 (SEQ ID NO:10): a BLASTP search of the NR database
with VH CDR3 as the query yielded no hits.

BLAST SEARCHES USING KABAT DATABASE

S2C6 VL (SEQ ID NO:2): a BLASTP search of the Kabat database using S2C6 VL as the query yielded no hits with 100% identity and numerous hits with 89-91% identity to the query. The first 5 are shown:

KADBID 005591, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (5-14...),
KADBID 005594, mouse IG KAPPA LIGHT CHAIN VARIABLE
10 REGION (10VA...),
KADBID 005593, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (12-4...),
KADBID 005603, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (17s....),
15 KADBID 005588, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (TEPC...).

VL CDR1 (SEQ ID NO:3): a BLASTP search of the Kabat database with VL CDR1 as the query yielded no hits with 100% identity and numerous hits with 93% identity (15/16). The first 5 are shown below:

KADBID 005720, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (BW24...),
KADBID 005614, mouse IG KAPPA LIGHT CHAIN VARIABLE
25 REGION (PME7...),
KADBID 005624, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (C5-7...),
KADBID 005621, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (40-6...),
30 KADBID 005640, mouse IG KAPPA LIGHT CHAIN VARIABLE
REGION (40-9...).

VL CDR2 (SEQ ID NO:4): a BLASTP search of the Kabat database with VL CDR2 as the query yielded no hits.

35

VL CDR3 (SEQ ID NO:5): a BLASTP search of the Kabat database with VL CDR3 as the query yielded 1 hit with 100% identity to the query:

5 KADBID 005681, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (NC10...).

S2C6 VH (SEQ ID NO:7): a BLASTP search of the Kabat database using S2C6 VH as the query yielded no hits with 100% identity and numerous hits with 79-85% identity to the query. The first 5 of the hits are shown below:

10 KADBID 001498, mouse IG HEAVY CHAIN VARIABLE REGION (HDEX24),
KADBID 001494, mouse IG HEAVY CHAIN VARIABLE REGION (HDEX5),
15 KADBID 001529, mouse IG HEAVY CHAIN VARIABLE REGION (163.72'CL),
KADBID 001500, mouse IG HEAVY CHAIN VARIABLE REGION (HDEX37),
KADBID 001597, mouse IG HEAVY CHAIN VARIABLE REGION (BB128'CL),
20

VH CDR1 (SEQ ID NO:8): a BLASTP search of the Kabat database with VH CDR1 as the query yielded no hits

25 VH CDR2 (SEQ ID NO:9): a BLASTP search of the Kabat database with VH CDR as the query yielded no hits with 100% identity and 10 hits with 87-88% identity to the query. The first 5 are shown:

30 KADBID 001535, mouse IG HEAVY CHAIN VARIABLE REGION (H10"CL),
KADBID 001534, mouse IG HEAVY CHAIN VARIABLE REGION (H81'CL),
KADBID 001533, mouse IG HEAVY CHAIN VARIABLE REGION (H50'CL),
35 KADBID 019741, mouse IG HEAVY CHAIN VARIABLE REGION (Clone F'CL),

KADBID 001529, mouse IG HEAVY CHAIN VARIABLE REGION
(163.72'CL),

VH CDR3 (SEQ ID NO:10): BLASTP search of the Kabat database
5 with VH CDR3 as the query yielded no hits.

7. EXAMPLE: BIOLOGIC ACTIVITY OF S2C6

7.1 MATERIALS AND METHODS

10 7.1.1 ANTI-CD40 ANTIBODY PREPARATION

The S2C6 hybridoma was cultured at 37°C in complete IMDM
(Gibco BRL, Grand Island, NY) containing 10% fetal bovine
serum (FBS), 100 units/ml penicillin and 100 mg/ml
streptomycin. The culture was harvested by centrifugation
15 and the supernatant was collected by filtration using a 0.2
micron filter. Subsequently the supernatant was loaded onto
a GammaBind™ Sepharose column (Pierce), washed with phosphate
buffered saline (PBS), and eluted with 0.1 M glycine pH 2.5.
Immediately upon elution, the antibody was neutralized with 1
20 M Tris pH 8.0, dialyzed into PBS, and filter sterilized. MAB
preparations were analyzed by size exclusion chromatography.
Only samples of greater than 99% monomeric protein were used
for the studies described herein.

25

7.1.2 HUMAN TUMOR XENOGRAFT MODELS

Ninety female C.B.-17 SCID mice were obtained (Taconic
Labs, Germantown, NY) at age 6 to 8 weeks and quarantined for
2 weeks. Control groups of mice were injected intravenously
(i.v.) with a human B cell tumor line: Ramos (non-Hodgkins
30 lymphoma), HS Sultan (multiple myeloma) or IM-9 (multiple
myeloma) cells (1×10^6 - 2×10^6 cells). The remaining mice were
divided into two groups; half were treated with 200 μ l of a
1:10 dilution of anti-asialo-GM1 (Wako Chemicals, Richmond,
VA) i.v., one day prior to the injection of tumor cells, to
35 remove host natural killer cells (Murphy et al., 1992, Eur.
J. Immunol. 22:241). Mice in the two groups were injected

i.v. with Ramos, HS Sultan or IM-9 cells (1×10^6 - 2×10^6 cells). Mice in the test groups were then injected intraperitoneally (i.p.) with 1 mg/kg of S2C6 IgG prepared as described in Section 7.1.2 starting on day 1 or day 5 post tumor implant, according to the following schedule and were monitored for partial paralysis or other signs of disease.

Xenograft Tumor Model Schedule

Group	Tumor cell line	Antibody (1mg/kg, i.p.)	Anti-asialo GM1	Days treated with mAb
1 Control	Ramos	-	-	-
2	Ramos	S2C6	-	1, 5, 9, 13, 17
3	Ramos	S2C6	-	5, 9, 13, 17, 21
4	Ramos	S2C6	+	1, 5, 9, 13, 17
5	Ramos	S2C6	+	5, 9, 13, 17, 21
6 Control	HS Sultan	-	-	-
7	HS Sultan	S2C6	-	1, 5, 9, 13, 17
8	HS Sultan	S2C6	-	5, 9, 13, 17, 21
9	HS Sultan	S2C6	+	1, 5, 9, 13, 17
10	HS Sultan	S2C6	+	5, 9, 13, 17, 21
11 Control	IM-9	-	-	-
12	IM-9	S2C6	-	1, 5, 9, 13, 17
13	IM-9	S2C6	-	5, 9, 13, 17, 21
14	IM-9	S2C6	+	1, 5, 9, 13, 17
15	IM-9	S2C6	+	5, 9, 13, 17, 21

7.1.3 PERIPHERAL BLOOD B CELL ISOLATION

Peripheral blood B cells were isolated by positive selection using immobilized antibodies against both CD19 and CD20. The final isolated cell population contained greater than 85% B cells as determined by flow cytometry. For storage, the cells were diluted to 4×10^7 cells/ml in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide and stored in a liquid nitrogen freezer.

7.1.4 B CELL PROLIFERATION ASSAY

Human peripheral blood B cells were thawed and incubated in 96-well tissue culture plates at 1×10^5 per well in IMDM medium plus 10% FBS in the presence of 5 ng/ml recombinant human IL-4 (Biosource) and various dilutions of an anti-CD40 mAb: S2C6, G28-5 (Bristol-Myers Squibb) or M3 (Genzyme #80-3702-04). As a control, cells were incubated with IL-4 and

- an irrelevant control mAb, EXA2-1H8 (anti-*Pseudomonas* exotoxin). The plates were incubated at 37°C for 3 days and then pulsed for 16 h with 0.5 mCi ³H-thymidine/well. Cells were harvested onto 96-well glass fiber filters using a
- 5 Filtermate 196 Harvester™ (Packard Instruments) and combined with scintillation fluid. The extent of ³H-thymidine incorporated into nascent DNA was measured by liquid scintillation counting using a Topcount LSC™ (Packard Instruments).
- 10 A Jurkat cell line selected to express constitutive high levels of CD40L ("Jurkat/CD40L"), was used as CD40L stimulator cells (Malik et al., 1996, J. Immunol. 156:3952-60). To eliminate proliferation of the stimulator cells, they were treated with mitomycin C (50 mg/ml) in PBS for 20
- 15 min at 37°C followed by 3 washes in PBS prior to combining with B cells. B cells (1x10⁶/well) were combined with Jurkat/CD40L cells and assayed as above. B cells and IL-4 were initially combined with stimulator cells (2.5x10⁴/well) directly followed by addition of the anti-CD40 mAbs.
- 20 Monoclonal antibodies were titrated with either a fixed concentration of stimulator cells or stimulator cells were titrated with a fixed concentration of mAb.

7.1.5 CD40/CD40L BINDING ASSAY

- 25 The Jurkat/CD40L cell line was used as a target cell line in these assays. Cells were adjusted to a density to 2x10⁷/ml at 50 µl per sample. Binding was performed in RPMI 1640 media (Gibco) + 10% FBS. To determine receptor saturation, Jurkat/CD40L cells were incubated with increasing
- 30 concentrations of CD40-Ig (a soluble fusion protein of CD40 and human immunoglobulin) (Noelle et al., 1992, Proc. Natl. Acad. Sci. USA 89:6550-6554), washed and incubated with fluorescein isothiocyanate conjugated to anti-human immunoglobulin ("FITC-anti-human Ig"). The resultant binding was evaluated using a FacScan™ flow cytometer (Becton
- 35 Dickinson). Recombinant soluble CD40-Ig (25 µg/ml) was pre-incubated for 1 h on ice with increasing concentrations of

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mAb S2C6. The anti-CD40 mAb G28-5; M3; and anti-*Pseudomonas* exotoxin, an isotype control, were used for comparison. The recombinant human soluble CD40 ligand (CD154-muCD8), produced as a fusion protein with murine CD8 and labeled with FITC, was obtained from Research Diagnostics, Inc. (Flanders, NJ). Dilutions of soluble CD40-Ig and anti-CD40 mAbs were made at a 4-fold final concentration, pre-incubated on ice for 1 h and then combined with Jurkat cells on ice for 1 h. Cells were washed and labeled with FITC-Goat anti-human F(ab')₂, (Jackson Labs, Fc-specific #109-096-098). The extent of CD40 binding was determined by flow cytometry.

7.2 RESULTS

~~7.2.1 IN-VITRO STUDIES: mAb S2C6 PROMOTES CD40/CD40L INTERACTION~~

15 To evaluate the effect of anti-CD40 mAbs on the binding of soluble CD40 to CD40L expressed on the surface of activated T cells, increasing concentrations of various CD40 mAbs were pre-incubated with 25 µg/ml soluble CD40-Ig followed by incubation of the complexes with Jurkat/CD40L cells. CD40L expression on selected CD40L⁺ Jurkat T cells was initially verified by flow cytometry with FITC-labeled anti-CD40L (data not shown). CD40 binding to CD40L on these target cells was then determined by flow cytometry of the Jurkat/CD40L cells using FITC-goat anti-human Ig to detect the bound CD40-Ig. Titration with CD40-Ig showed receptor saturation at approximately 25 µg/ml CD40-Ig. Using saturating concentrations of soluble CD40, S2C6 complexed with CD40 at ratios ranging from 0.25 to 2:1 (mass:mass) resulted in a dose-dependent increase in CD40 binding to CD40L (approximately 50%, 100%, 146% and 220% at concentrations of approximately 6 µg/ml, 13 µg/ml, 25 µg/ml, and 50 µg/ml, respectively) (Figure 4). A similar titration with the inhibitory antibody M3 blocked CD40/CD40L binding in a dose dependent manner. mAb G28-5 showed no effect of CD40/CD40L binding at concentrations up to 25 µg/ml and was

level (30 ng/ml) of the various mAbs. B cell activation in response to treatment was then measured by ³H-thymidine incorporation at 72 h post-stimulus. T cell titration in the presence of mAb M3 resulted in B cell proliferation similar to that seen with control Ig (Figure 6).

Although mAb G28-5 provided some B cell activation in the absence of ligand (Figure 7), CD40L⁺ T cell titration in the presence of G28-5 only nominally increased B cell proliferation (1.3-fold) over the level seen with G28-5 alone. In contrast, B cell proliferation increased in the presence of S2C6 in a dose dependent manner with increasing numbers of T cell stimulator cells to 3-fold above mAb-only stimulation with a B cell to T cell stimulator ratio of 4:1.

These data demonstrate that unlike M3 and G28-5, S2C6 can surprisingly synergize with CD40L to promote B cell proliferation via CD40.

In a second assay of this type, B cells were either titrated with an anti-CD40 mAb or combined with non-proliferating CD40L⁺ T stimulator cells at a fixed ratio of 4:1 (B:T) and titrated with an anti-CD40 mAb (Figure 7).

These results demonstrate that, under these conditions, activation of primary human peripheral blood B cells increased 2-fold at 10 µg/ml of mAb G28-5 and ligand, as compared to G28-5 alone. To a surprising degree, S2C6 was significantly more active and in the presence of ligand increased B cell proliferation in a dose dependent manner to 16.2-fold at 10 µg/ml (the highest level tested) as compared to S2C6 alone.

Taken together, these data indicate that S2C6 complexed to CD40 increases CD40L binding. Although S2C6 by itself will stimulate B cell proliferation in a manner similar to G28-5, S2C6 is distinguished from G28-5 by its ability to increase CD40L binding and the subsequent magnitude of the CD40L-mediated activation signal.

8. EXAMPLE: MONOCLONAL ANTIBODY S2C6
INHIBITS TUMOR GROWTH

To evaluate the anti-tumor activity of native mAb S2C6, female C.B.-17 SCID mice were divided into two groups (20 mice/group). Half of the mice of each group were treated with anti-asialo-GM1 to blunt host natural killer cell activity (Murphy et al., 1992, Eur. J. Immunol. 22:241). The following day, mice were injected i.v. with Ramos, HS Sultan or IM-9 cells (1×10^6 cells). Mice were then injected i.p. with 1 mg/kg of mAb S2C6 IgG, as described in Materials and Methods in Section 7 *supra* and monitored for partial paralysis or other signs of disease onset.

Monoclonal antibody S2C6 treatment of animals harboring Ramos human B cell lymphoma (Figure 8A), HS Sultan multiple myeloma (Figure 8B), or IM-9 multiple myeloma (Figure 8C), resulted in significant reduction in tumor mass and subsequent tumor-related morbidity and mortality. In parallel studies, efficacy was sustained in the presence of anti-asialo-GM1, suggesting that the increased survival in the presence of mAb S2C6 was not due to nonspecific NK activity. The IM-9 cell line is an aggressive tumor model that, like multiple myeloma, secretes human Ig as a surrogate marker of disease.

Treatment of IM-9 diseased mice with mAb S2C6 significantly increased animal survival. These studies clearly demonstrate that S2C6 has potent anti-tumor activity against engrafted human tumors in mice.

9. EXAMPLE: A SINGLE-CHAIN ANTI-CD40 IMMUNOTOXIN
FUSION PROTEIN BINDS CD40-Ig

BD1-S2C6 sFv (single-chain anti-CD40 immunotoxin, a fusion protein consisting of the amino acid sequence of bryodin 1 (BD1) (Francisco et al., 1997, J. Biol. Chem. 272(39):24165-24169) fused to the variable regions of monoclonal antibody S2C6) was expressed in E. coli as inclusion bodies, denatured and refolded.

Briefly, total RNA was isolated from S2C6 hybridoma cells using TRIZOL reagent (Life Technologies) following the

guanidine at 5 mg/ml, refolded by rapid dilution (1:100) into PBS containing 0.3M L-arginine and 2 mM DTT, and dialyzed against 20 mM sodium phosphate buffer, pH 7.4, for subsequent purification.

- 5 The refolded protein was isolated using Blue Sepharose followed by affinity chromatography over immobilized CD40-Ig.

The purified protein was then tested for binding to immobilized CD40-Ig in ELISA. Microtiter plates were coated with CD40-Ig at 0.5 µg/ml followed by the addition of dilutions of purified BD1-S2C6 sFv in PBS (pH 7.4) with 1% bovine serum albumin and 0.05% Tween-20 in the presence of 25 µg/ml S2C6 mAb (▲), 25 µg/ml control antibody BR96 (●), or no excess antibody (■). Binding of BD1-S2C6 sFv to the immobilized receptor was detected by the addition of BD1-specific rabbit antiserum (Seattle Genetics, Inc., Bothell, Washington) followed by the addition of horseradish peroxidase conjugated goat anti-rabbit Ig.

- 15 The binding of BD1-S2C6 sFv to CD40-Ig was completely inhibited by the addition of excess S2C6 mAb but not by the addition of the control mAb (Figure 9).

20 10. DEPOSIT OF ~~MICROORGANISM~~ *Hybridoma*

- a* Hybridoma S2C6, secreting native monoclonal antibody S2C6, was deposited on May 25, 1999, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassass, Virginia 20110-2209, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedures, and assigned accession number PTA-110.

- 30 11. SPECIFIC EMBODIMENTS, CITATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such

modifications are intended to fall within the scope of the appended claims.

Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of which are incorporated herein by reference in their entireties.

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